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Sodium Ion Transport Decarboxylases and Other Aspects of Sodium Ion Cycling in Bacteria

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INTRODUCTION

In the energy metabolism of procaryotes, as well as of other cells, chemiosmotic phenomena are of central importance. As postulated by Mitchell (140–142, 144), an electrochemical gradient of protons is built at the expense of chemical or light energy by one of the proton pumps present in the bacterial membrane, e.g., respiratory chains, H^+-translocating adenosine triphosphatase (ATPase), photosynthetic reaction centers, or bacteriorhodopsin. The electrochemical proton gradient can then be used by the cell to drive energy-consuming processes, such as solute uptake, flagellar motion, or the synthesis of adenosine triphosphate (ATP) from adenosine diphosphate (ADP) and inorganic phosphate. Exergonic and endergonic reactions are thus coupled via the circulation of protons across the membrane.

Table 1 summarizes the energy conversion mechanisms that have been recognized in bacterial membranes. Although H^+ is the central coupling ion in bacterial energy metabolism, it is not the only one. For example, (i) halorhodopsin functions as an electrogenic chloride pump in *Halobacterium halobium* (171). (ii) Evidence for ATP-driven Na^+ and K^+ transport has been obtained, e.g., in *Streptococcus faecalis* (77, 89), *Propionigenium modestum* (83), and *Escherichia coli* (54, 55, 214). (iii) We have detected the Na^+-pumping oxaloacetate decarboxylase of *Klebsiella pneumoniae* (38, 39, 41, 45, 46) and *Salmonella typhimurium* (45) and methylmalonyl-coenzyme A (CoA) decarboxylase of *Veillonella alcalescens* (79–82) and *P. modestum* (83) that perform Na^+ extrusion at the expense of the free energy of decarboxylation reactions. Related Na^+-pumping decarboxylases are the glutaconyl-CoA decarboxylases of *Acidaminococcus fermentans*, *Peptostreptococcus asaccharolyticus*, and *Clostridium symbiosum* (20, 24, 25). (iv) As shown by Tokuda and Unemoto (200, 201), the marine alkalitolerant bacterium *Vibrio alginolyticus* uses respiration-derived energy to drive an electrogenic extrusion of Na^+ at alkaline pH. Next to H^+, Na^+ is the most important cation that by circulating over the bacterial membrane connects endergonic and exergonic processes. In this article I review recent developments of our own and other laboratories on Na^+-coupled bioenergetics in bacteria. For previous reviews on this topic, see references 41, 45, 119, 179, and 180.

Like other living cells, bacteria tend to establish gradients of Na^+ and K^+ ions between their cytoplasm and the surrounding medium such that the K^+ concentration of the cytoplasm is greater than, and the Na^+ concentration is less than, that of the environment (72). Whereas the high K^+ gradients appear to be essential, Na^+ gradients seem to be important only in special organisms or under special growth conditions. Neutrophilic bacteria living in fresh water do not generally require Na^+ ions for growth beyond the residual Na^+ that is invariably present in the medium (up to 0.1 mM from chemicals and glassware) (134).

On the other hand, Na^+ ions are essential for growth of marine (164), halophilic (52), and certain alkalophilic (14, 113, 114) and rumen (26) bacteria that live in Na^+-rich habitats. Sodium ions are also an important growth factor for methanogenic bacteria (160, 161) and for freshwater organisms when special substrates are utilized (154, 155, 212). Why is Na^+ required by these bacteria? At least four different functions of Na^+ for bacterial cells are known: (i)

TABLE 1. Energy conversion mechanisms in bacterial membranes

Process	Example	Energized state/energy source
Energy releasing (primary energy source)		
Light	Photosynthesis	$\Delta\tilde{\mu}H^+$
	Photocycle (bacteriorhodopsin)	$\Delta\tilde{\mu}H^+$
	Photocycle (halorhodopsin)	$\Delta\tilde{\mu}Cl^-$
Chemical energy	Repsiratory chains	$\Delta\tilde{\mu}H^+$, $\Delta\tilde{\mu}Na^+$
	Other oxidoreductions with organic or inorganic compounds	$\Delta\tilde{\mu}H^+$
	ATP hydrolysis	$\Delta\tilde{\mu}H^+$, $\Delta\tilde{\mu}Na^+$, $\Delta\tilde{\mu}K^+$
	Decarboxylation	$\Delta\tilde{\mu}Na^+$
Energy consuming		
Osmotic work	Solute uptake	$\Delta\tilde{\mu}H^+$, $\Delta\tilde{\mu}Na^+$
Mechanical work	Flagellar motion	$\Delta\tilde{\mu}H^+$, $\Delta\tilde{\mu}Na^+$
Chemical work	ATP synthesis	$\Delta\tilde{\mu}H^+$, $\Delta\tilde{\mu}Na^+$
	Reduction of NAD^+[a]	$\Delta\tilde{\mu}H^+$

[a] NAD^+, Oxidized nicotinamide adenine dinucleotide.

sodium ion-solute cotransport systems (119), (ii) sodium-coupled energy conservation and energy transduction (41, 45, 83, 180, 201), (iii) pH homeostasis mechanisms (14, 159), and (iv) activation of special enzymes (24, 44, 80, 184, 209). Since it is enzymes catalyzing Na^+ translocation that are specifically activated, all of the functions of Na^+ are related to transport across the membrane. It is clear, therefore, that movement of Na^+ ion across the membrane is an essential physiological function of these bacteria. It is also clear that import and export systems must exist that balance the fluxes of Na^+ ions. In many cases, the essentiality of Na^+ for growth can be attributed to its role in the uptake of solutes (119). Also, alkalophilic strains of *Bacillus* spp. (86, 87, 106) and *Vibrio alginolyticus* (30, 36) use the Na^+ gradient to drive flagellar movement, and *P. modestum* uses this energy source for ATP synthesis (83). The export of Na^+ from the cytoplasm against the concentration gradient is accomplished by either an Na^+/H^+ antiporter (111) driven by the electrochemical H^+ gradient or an Na^+ pump driven by decarboxylation (24, 38, 79), ATP hydrolysis (9, 10, 76–78, 105), or reduced nicotinamide adenine dinucleotide (NADH) oxidation (201, 202). Thus, in a number of bacteria, endergonic and exergonic membrane reactions are linked by the cycling of Na^+ ions. Such systems are summarized in Fig. 1 and are described in more detail below.

FORMATION OF AN Na^+ GRADIENT IN BACTERIA BY PRIMARY SODIUM PUMPS

Sodium Ion Transport Decarboxylases

The utilization of energy from decarboxylations to drive an active transport of Na^+ ions is a new concept of biological energy conservation that was detected during studies of oxaloacetate decarboxylase of *Klebsiella pneumoniae* induced for citrate fermentation (38).

Citrate metabolism by bacteria. The facultative anaerobic bacteria *K. pneumoniae* and *Salmonella typhimurium* and many other species of the *Enterobacteriaceae* can grow aerobically or anaerobically, utilizing citrate as the sole carbon source. *E. coli*, on the other hand, cannot utilize citrate as a sole source of carbon and energy (108). This inability of *E. coli* to grow on citrate is widely used as a characteristic for bacterial diagnostics (136). The failure of *E. coli* to degrade exogenous citrate under aerobic conditions can be attributed to its lack of a citrate transport system: the cells contain the enzymes of the tricarboxylic acid cycle that degrade citrate (123). A number of investigators have isolated citrate-positive *E. coli* strains; the Cit^+ phenotype can be attributed to plasmids that encode citrate transport (90, 91, 165, 181). Sequence homologies were found among different Cit^+ plasmids (92, 169) and between plasmids and the DNA of several enterobacterial species that are phenotypically Cit^+ (85). In addition, a complex chromosomal mutation enabling citrate utilization in *E. coli* was isolated and characterized (69). It was proposed that a silent citrate transport gene became activated by the mutation (69).

The barrier to expression of the proteins required for citrate transport is also overcome under anaerobic conditions when *E. coli* metabolizes exogenous citrate if a second growth substrate, e.g., glucose, is present (128). Such cells contain large amounts of citrate-lyase by which citrate is cleaved to acetate and oxaloacetate (31, 34, 213). The latter is subsequently reduced to succinate by reducing equivalents provided by the cosubstrate (128). This reduction is accompanied by energy conservation at the fumarate reductase step (109). *Proteus rettgeri* also conserves energy by this mechanism, but it uses a modified pathway for the anaerobic breakdown of citrate, in which the reducing power for converting oxaloacetate to succinate is obtained by oxidizing some citrate via the oxidative branch of the tricarboxylic acid cycle (110), including the step catalyzed by 2-oxoglutarate dehydrogenase. Since the expression of this enzyme is repressed in *E. coli* under anaerobic conditions (2), a cosubstrate is required by this bacterium to provide reducing power for succinate formation from citrate (128).

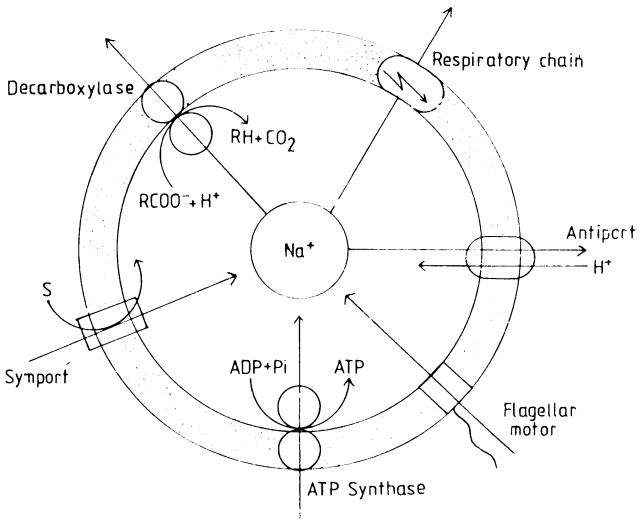


FIG. 1. Summary of systems performing energy coupling by sodium circulation in bacteria. Sodium transport decarboxylases and Na^+-coupled ATP synthase exist in anaerobic bacteria, a respiratory Na^+ pump occurs in marine organisms, Na^+-driven flagellar motors were found in marine and alkalophilic species, and Na^+/H^+ antiport and Na^+-symport systems are widely distributed.

A different citrate fermentation pathway occurs in *K. pneumoniae* and *Salmonella typhimurium* (17, 31, 154, 155). These bacteria lack a membrane-bound fumarate reductase and thus lack the enzymatic equipment to conserve energy by electron transport-linked phosphorylation. Instead, the oxaloacetate produced by citrate-lyase is decarboxylated to pyruvate (31, 184), which is subsequently split to acetyl-CoA and formate by pyruvate formate-lyase. Acetyl-CoA is converted to acetyl phosphate from which ATP is formed by substrate-level phosphorylation (Fig. 2) (49). Thus, only about 1 mol of ATP is synthesized per mol of citrate utilized. However, additional energy is conserved in these bacterial cells by developing an Na^+ gradient at the expense of oxaloacetate decarboxylation (38), as is described next.

Characterization of oxaloacetate decarboxylase as an Na^+ pump. Oxaloacetate decarboxylase is a key enzyme of citrate fermentation in *K. pneumoniae* or *Salmonella typhimurium* (152–156). Remarkable properties of the crude *K. pneumoniae* enzyme were reported by Stern (184). The decarboxylase was associated with the particulate material of the cell, required Na^+ (but no divalent cations) for catalytic activity, and was inhibited by avidin, indicating that biotin is involved in the catalysis. These properties would be quite unusual if the only function of the enzyme were catalysis of a step in the pathway of citrate degradation. The membrane-linked character of the enzyme and its activation by Na^+ were reminiscent, however, of transport enzymes such as the Na^+/K^+ ATPase and suggested to us that oxaloacetate decarboxylase might act as an Na^+ pump (38, 39). This hypothesis was further supported by a negative free energy change of the decarboxylation reaction of about 29 kJ/mol, which would be sufficient to drive uphill transport of Na^+ ions.

The function of oxaloacetate decarboxylase as an Na^+ pump was demonstrated with inverted vesicles of *K. pneumoniae* prepared with a French pressure cell (Fig. 3).

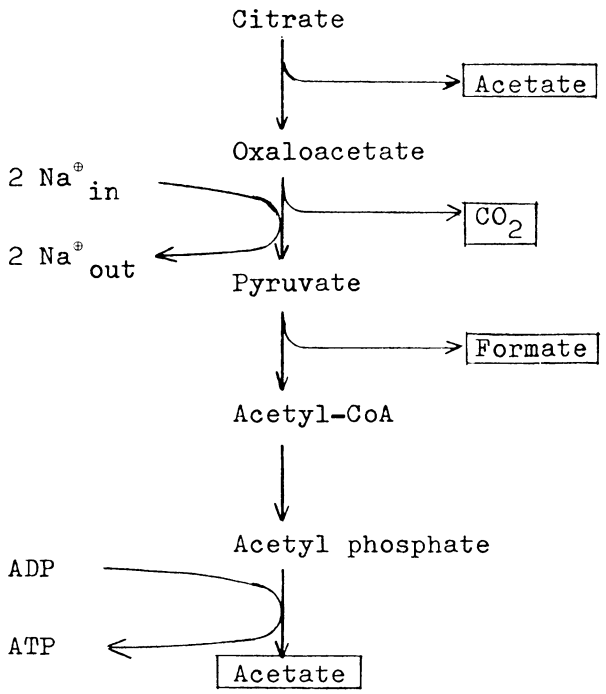


FIG. 2. Citrate fermentation pathway of *K. pneumoniae*. (Reproduced from reference 49 with permission.)

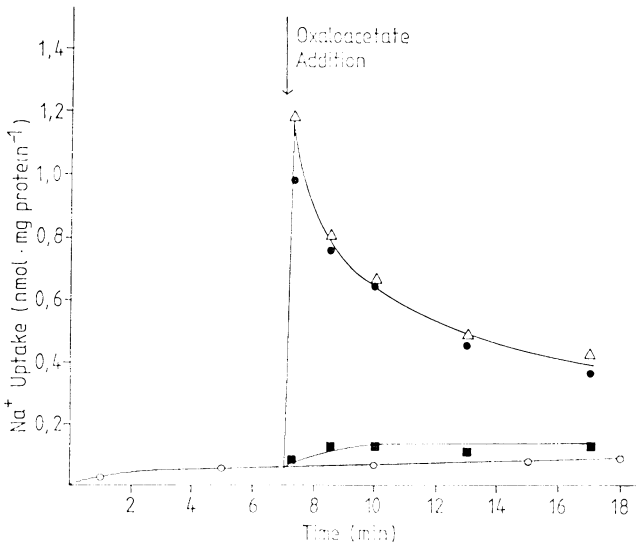


FIG. 3. Kinetics of Na^+ uptake into French press vesicles from *K. pneumoniae*. The transport was initiated by oxaloacetate addition as indicated (●). Symbols: Vesicles preincubated with avidin (■); vesicles preincubated with avidin-biotin complex (△); control without oxaloacetate addition (○). (Reproduced from reference 45 with permission.)

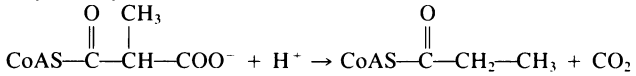
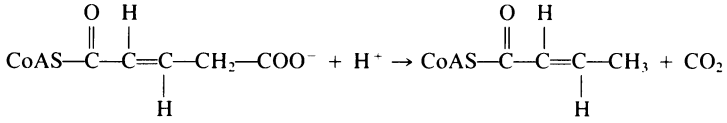
Sodium ions were not accumulated inside these vesicles to an appreciable level in the absence of oxaloacetate (38, 43). Upon oxaloacetate addition, the internal Na^+ concentration increased rapidly. This was followed by a slower efflux after the oxaloacetate had been completely decarboxylated by the highly active decarboxylase in these vesicles. Whether the efflux occurs through leaks in the membrane or through another porter is unknown.

The free energy of a decarboxylation reaction is used to drive an active transport of Na^+ ions, a completely unexpected and new type of conversion of chemical energy which exists in addition to the well-known energy conservation mechanisms of electron transport and substrate-level phosphorylation (193).

It should be emphasized that all living cells use the free energy of decarboxylation reactions to drive certain otherwise unfavorable biosynthetic reactions. Prominent examples are the biosyntheses of fatty acids, phosphoenolpyruvate, sphingosine, and δ-aminolevulinate which are driven by the decarboxylation of malonyl-CoA, oxaloacetate, serine, and glycine, respectively. However, the conversion of decarboxylation energy into another energy source ($\Delta\tilde{\mu}Na^+$) by an oxaloacetate decarboxylase Na^+ pump is quite different from the decarboxylation-derived promotion of the biosynthetic reactions listed above.

If the physiological function of oxaloacetate decarboxylase in intact cells is catalysis of an export of Na^+ ions, decarboxylase with an inverted orientation in the membrane should catalyze Na^+ uptake. A predominantly inverted orientation of *E. coli* vesicles prepared with a French press has been reported (62). The orientation of oxaloacetate decarboxylase in French press vesicles from *K. pneumoniae* was inferred from the amount of catalytic activity which could be blocked by avidin treatment. Inhibitable enzyme molecules should have the biotin prosthetic group facing the outside, an inverted orientation compared with that in whole cells. By this method it was found that about 55% of the total oxaloacetate decarboxylase in the vesicles was in the in-

TABLE 2. Sodium ion transport decarboxylases

Type of decarboxylase	Organism	Fermentation pathway
Oxaloacetate 	*Klebsiella penumoniae*	Citrate → acetate
	Salmonella typhimurium	Citrate → acetate
Methylmalonyl-CoA 	*Veillonella alcalescens*	Lactate → propionate
	Propionigenium modestum	Succinate → propionate
Glutaconyl-CoA 	*Acidaminococcus fermentans*	Glutamate → butyrate
	Peptostreptococcus asaccharolyticus	Glutamate → butyrate
	Clostridium symbiosum	Glutamate → butyrate

verted orientation (41). By blocking the outwardly oriented decarboxylase molecules with avidin, the Na^+ pump activity was simultaneously abolished, whereas avidin that had been pretreated with an excess of biotin was without effect (43).

The kinetics of and the effect of inhibitors and ionophores on Na^+ transport have been studied in some detail. A characteristic feature of the process is the low degree of its coupling to decarboxylation; in the uninhibited system only about 7 nmol of Na^+ was transported into vesicles by decarboxylation of 480 nmol of oxaloacetate (43). The rate of Na^+ uptake decreased in parallel with reducing the rate of oxaloacetate decarboxylation caused by slow generation of oxaloacetate enzymatically, lowering the temperature, or inhibiting the decarboxylase with oxalate (43). However, as the rate decreased, the efficiency of the Na^+ pump increased considerably: the internal Na^+ concentrations generated by retarding transport increased two- to fourfold and the duration of transport increased from <1 min to 15 min. During this time the steady-state concentration of internal Na^+ was constant; thus, Na^+ efflux was compensated by active influx.

When valinomycin and K^+ were present, about twice as much Na^+ was accumulated in the vesicles even under conditions of fast oxaloacetate decarboxylation (40, 43). These results are consistent with the operation of an electrogenic Na^+ transport which creates an inside-positive membrane potential and limits Na^+ uptake. Assuming an electrical capacitance of the membrane of 1 $\mu F/cm^2$ (141), a spherical shape of the vesicles with a diameter of 120 nm (163), and a ratio of 1.1-μl internal volume per mg of membrane protein (43), the transport of 5-pmol charges per mg of membrane protein will change the membrane potential by 1 mV. To build up the experimentally observed membrane potential of 65 mV (43) would require transport of 0.32-nmol charges per mg of membrane protein, which would be the upper limit of Na^+ concentration obtainable without charge balancing. This concentration is well below the experimentally determined value of about 1 nmol/mg in the nonretarded system (Fig. 3); the discrepancy indicates the significance of the membrane potential as a barrier to the uptake of Na^+ ions. The amount of Na^+ uptake, therefore, increased when the membrane potential was discharged, e.g., with valinomycin and K^+ (43). Similarly, it has been shown that, by retarding the decarboxylation rate, secondary ion fluxes dissipate the membrane potential before the substrate is completely consumed, thus accounting for the observed increase of Na^+ uptake (43). As expected, no Na^+ gradient was developed in the presence of the Na^+-carrying ionophore monensin or nigericin, whereas the proton ionophore trifluoromethoxycarbonylcyanide *p*-phenylhydrazone was without effect on the kinetics of Na^+ uptake into bacterial vesicles. The pumping of Na^+ by oxaloacetate decarboxylase is, therefore, a primary event, not a secondary event mediated by an Na^+/H^+ antiporter.

The magnitude of the membrane potential generated by the Na^+-pumping oxaloacetate decarboxylase has been determined from the distribution of the lipophilic anion $[^{14}C]SCN^-$ between interior and exterior vesicular spaces (43). At 0°C and a high concentration of oxaloacetate (which enabled the pump to operate for >20 min), a membrane potential of 65 mV was developed 1.5 min after oxaloacetate addition; the potential subsequently gradually declined. Concomitantly with development of the membrane potential, an Na^+ concentration gradient (ΔpNa^+) with a stable value of 49 mV was created. Thus, the total sodium motive force ($\Delta\tilde{\mu}Na^+$), which according to the chemiosmotic theory is the sum of electrical and chemical potential, amounts to 114 mV (equation 1).

$$\Delta\tilde{\mu}Na^+ = \Delta\psi - \frac{2.3\ RT}{F}\Delta pNa^+ \quad (1)$$

Other biotin-dependent sodium ion transport decarboxylases. As well as the oxaloacetate decarboxylase, a number of others have been recognized as sodium-pumping enzymes; these are summarized in Table 2. Also listed are the organisms in which these enzymes have been found and the fermentation pathways in which they participate.

Oxaloacetate decarboxylase of *Salmonella typhimurium*. Like *K. pneumoniae*, *Salmonella typhimurium* can grow on citrate under aerobic or anaerobic conditions (154). Aerobically grown cells contain all enzymes of the tricarboxylic acid cycle as well as those of the citrate fermentation pathway, citrate-lyase and oxaloacetate decarboxylase (154). Two types of decarboxylases are present, a soluble enzyme that is inhibited by ethylenediaminetetraacetic acid

and activated by Mn^{2+}, and a membrane-bound enzyme that is inhibited by avidin and activated by Na^+, thus resembling the Na^+-dependent oxaloacetate decarboxylase of *K. pneumoniae* (154). Anaerobic growth of uninduced cells on citrate started only after a lag of 20 to 40 h and was completely dependent on added Na^+ (unpublished results). The content of the Na^+-dependent oxaloacetate decarboxylase was considerably higher in anaerobically than in aerobically grown cells (unpublished results). The pathways of anaerobic citrate metabolism by *Salmonella typhimurium* and *K. pneumoniae* are the same, but unlike *K. pneumoniae*, the aerobic growth of *Salmonella typhimurium* on citrate was strictly Na^+ dependent (optimum concentration, 7 to 10 mM [154]), whereas growth on glucose, glycerol, or L-malate was Na^+ independent. Since citrate degradation can proceed aerobically via the tricarboxylic acid cycle (for which Na^+ is not required), the Na^+ requirement might reflect a necessity to activate the Na^+-pumping oxaloacetate decarboxylase which might have an essential function in these bacteria, or Na^+ might be required for citrate transport. Citrate transport into aerobically grown *Salmonella typhimurium* is in fact stimulated by Na^+ (100). A Na^+-independent citrate transport system which is expressed in *K. pneumoniae* during aerobic growth on citrate (49) may therefore be lacking in *Salmonella typhimurium*.

Methylmalonyl-CoA decarboxylase. Methylmalonyl-CoA decarboxylase, another Na^+-transporting decarboxylase (79), was detected in the strictly anaerobic bacterium *V. alcalescens* (63, 64), which grows at the expense of the fermentation of lactate to acetate, propionate, CO_2, and H_2, according to the equation, 2 lactate + ADP + $P_i \rightarrow$ acetate + propionate + CO_2 + H_2 + ATP. By this pathway (57, 95, 148, 167), one-half of the lactate pool is oxidized to pyruvate and then carboxylated to oxaloacetate, which by malate-lactate transhydrogenase (1) (equation 2) is reduced to malate. The other half of the lactate pool is oxidized to pyruvate which, upon degradation to acetate, CO_2, and H_2, generates 1 mol of ATP by substrate-level phosphorylation.

$$\text{lactate} + \text{oxaloacetate} \rightleftharpoons \text{pyruvate} + \text{malate} \quad (2)$$

The malate is reduced to succinate by the reducing equivalents obtained from the initial oxidation of lactate: this process generates an additional ATP in the fumarate reductase step (35). The succinate is converted to succinyl-CoA, followed by rearrangement to (*R*)-methylmalonyl-CoA and isomerization to (*S*)-methylmalonyl-CoA. The latter is decarboxylated to propionyl-CoA, and finally CoA transfer to succinate regenerates succinyl-CoA and produces propionate as an end product (Fig. 4) (64).

The fermentation of lactate by propionibacteria also involves the degradation of succinate to propionate. In contrast to *V. alcalescens*, these bacteria possess a transcarboxylase (221) which links the exergonic decarboxylation of methylmalonyl-CoA to the endergonic carboxylation of pyruvate to oxaloacetate. Attempts to demonstrate a similar transcarboxylase in *V. alcalescens* have failed (64). This organism contains instead distinct enzymes for the carboxylation and decarboxylation reactions. Nonetheless, another type of coupling has been developed in this bacterium: the free energy of methylmalonyl-CoA decarboxylation is converted into an Na^+ gradient (79).

Initial studies on impure methylmalonyl-CoA decarboxylase showed that the activity was inhibited by avidin (63, 64). The decarboxylase was bound to particles that initially were assumed to be ribosomes (63, 64). Upon resolution of the particulate cell fraction of *V. alcalescens* by gel chromatography, membranes and ribosomes were resolved, and the decarboxylase was found to be associated with membranes, not ribosomes (80). This enzyme, like oxaloacetate decarboxylase, was specifically activated by Na^+ ions and functioned as an Na^+ pump as shown by the uptake of $^{22}Na^+$ into French press vesicles of *V. alcalescens* (80). (*S*)-Methylmalonyl-CoA was the better substrate for the decarboxylase, with an apparent K_m of 7 μM, but malonyl-CoA was also decarboxylated (apparent K_m, 35 μM) (80). Since acetyl-CoA can be more sensitively determined than propionyl-CoA, malonyl-CoA has been used as substrate for the decarboxylase in certain experiments (see below). Another methylmalonyl-CoA decarboxylase acting as an Na^+ pump was found in *P. modestum* (83), a strictly anaerobic bacterium which grows with succinate as sole source of carbon and energy (170). The succinate is converted to propionate and CO_2 by the same reactions by which succinate is degraded in *V. alcalescens* (Fig. 4) (83). The energy metabolism of *P. modestum* involves neither electron transport nor substrate-level phosphorylation and is entirely dependent on the Na^+ gradient generated by decarboxylation of methylmalonyl-CoA (83).

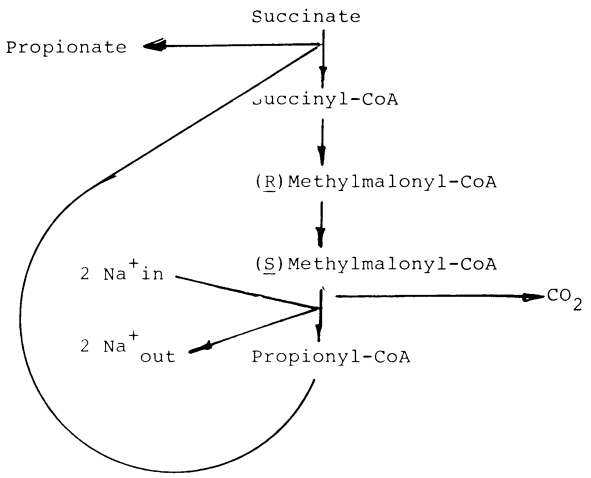


FIG. 4. Degradation of succinate to propionate and CO_2 by *V. alcalescens* and *P. modestum*.

Glutaconyl-CoA decarboxylase. Several obligately anaerobic bacteria ferment glutamate to ammonia, carbon dioxide, acetate, butyrate, and H_2. Two entirely different metabolic pathways for doing so are known, the methylaspartate and hydroxyglutarate pathways (22). The hydroxyglutarate pathway is used by *A. fermentans*, *Peptostreptococcus asaccharolyticus*, and *C. symbiosum* (22, 219). By this pathway, glutamate is converted via oxoglutarate to 2-hydroxyglutarate. Then, 2-hydroxyglutaryl-CoA is formed, which is dehydrated to glutaconyl-CoA by a complex reaction mechanism involving radicals (177): it is not chemically feasible to remove a hydroxyl ion from the carbon adjacent to the thioester group by an ionic mechanism. A similar reaction, probably also occurring by a radical mechanism, is the dehydration of lactyl-CoA to acrylyl-CoA in alanine fermentation by *C. propionicum* (117, 176).

The glutaconyl-CoA is decarboxylated to crotonyl-CoA, which dismutates to yield butyrate and acetate, thereby generating ATP by substrate-level phosphorylation (22, 219). The glutaconyl-CoA decarboxylase was detected by Buckel and Semmler (24, 25) in *A. fermentans* as a new biotin-dependent enzyme. Like other decarboxylases of this type,

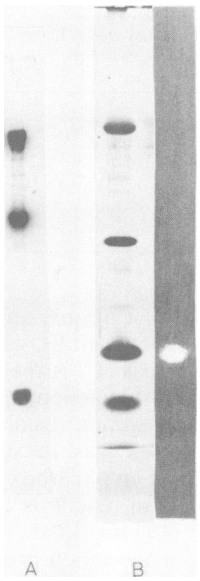


FIG. 5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of sodium ion transport decarboxylases. (A) Oxaloacetate decarboxylase of *K. pneumoniae*. (B) Methylmalonyl-CoA decarboxylase of *V. alcalescens*. (Far right) Western blot electrophoresis stained with fluorescein isothiocyanate-labeled avidin for detection of the biotin-containing subunit. (Reproduced from references 48 and 80 with permission.)

glutaconyl-CoA decarboxylase is firmly bound to the membrane, is activated by Na^+ ions, and functions as an electrogenic sodium pump (24, 25). The dependence of glutaconyl-CoA decarboxylase activity on the presence of Na^+ explains the previously observed requirement for this ion for glutamate fermentation by *Peptostreptococcus asaccharolyticus* (212). Another Na^+-dependent step in the same organism may be the uptake of glutamate into the cells. In *E. coli*, a cotransport of glutamate with Na^+ and H^+ ions has been demonstrated (58–60, 73, 130).

From growth yield studies it was concluded that in *Peptostreptococcus asaccharolyticus* and *C. symbiosum* the Na^+ gradient generated by glutaconyl-CoA decarboxylase substantially contributes to the energy budget of these cells. Sublethal doses of monensin reduced the growth yield of *Peptostreptococcus asaccharolyticus* to about half (219). Since this ionophore catalyzes an electroneutral Na^+/H^+ exchange, these results indicate that Na^+ and H^+ gradients are not equivalent forms of energy conservation in *Peptostreptococcus asaccharolyticus*. Glutamate fermentation in *A. fermentans* proceeds by the same pathway as in *Peptostreptococcus asaccharolyticus*. The two bacteria, however, have a different energy economy because the growth yield of *A. fermentans* is only about half that of *Peptostreptococcus asaccharolyticus* and about equal to monensin-treated *Peptostreptococcus asaccharolyticus* cells (219).

Purification and molecular properties of sodium transport decarboxylases. The purification of the various biotin-dependent sodium ion transport decarboxylases has been accomplished by very similar protocols (20, 42, 46, 82). The bacteria were disrupted with a French pressure cell, the membranes were isolated by high-speed centrifugation, and the enzyme was solubilized with a nonionic detergent, usually Triton X-100, in the presence of a high salt concentration (usually 0.5 M NaCl). The membrane extract was then applied to a monomeric avidin-Sepharose affinity column, and after contaminating proteins had been washed off the column, the decarboxylase was eluted with a biotin-containing buffer. The enzyme samples thus obtained were of high purity.

The subunit composition of the decarboxylases has been determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The patterns obtained for oxaloacetate decarboxylase and methylmalonyl-CoA decarboxylase (Fig. 5) (48, 80) indicate that these enzymes consist of three or four different subunits. The molecular weights of the polypeptides of all sodium transport decarboxylases analyzed are summarized in Table 3. Several homologies between these enzymes are apparent: all have one subunit in the range of M_r 60,000 to 65,000 and another in the range of M_r 33,000 to 35,000. In oxaloacetate and methylmalonyl-CoA decarboxylases a small subunit of M_r 10,500 to 14,000 has been observed consistently. In glutaconyl-CoA decarboxylase a polypeptide of similar size was found only occasionally, and the question has therefore been raised as to whether the small subunit is a proteolytic artifact (23). In the case of oxaloacetate and methylmalonyl-CoA decarboxylases, the questionable polypeptide would have to be formed prior to the affinity chromatography because the pattern of subunits of the pure enzymes was not significantly affected by storage. The small subunit does not contain biotin and therefore must have an affinity to another decarboxylase subunit to be retained on the column. Furthermore, tryptic hydrolysis of methylmalonyl-CoA decarboxylase yielded a number of small polypeptides but no specific cleavage to one with the molecular mass of the γ-chain (unpublished results). Finally, upon reconstitution of methylmalonyl-CoA decarboxylase, all four subunits including the γ-chain were incorporated into liposomes (81). From these observations it appears unlikely that the small subunit is an accidental contaminant, but final proof for its being a part of the decarboxylase probably requires elucidation of its function.

A marked difference among the various decarboxylases is the attachment site of the prosthetic group, biotin. In oxaloacetate decarboxylase it is bound to the large subunit (50), while methylmalonyl-CoA decarboxylase has a distinct biotin-containing subunit of small size (80). Biotin carrier proteins of similar size were also found in glutaconyl-CoA decarboxylase of *Peptostreptococcus asaccharolyticus* and *C. symbiosum*, whereas a group of very large polypeptides (M_r, 120,000 to 140,000) provided the biotin-containing subunits of *A. fermentans* (25). It is remarkable that the sodium transport decarboxylases, which have three different substrate specificities and are found in bacteria that are not closely related phylogenetically, share a similar composition of α and β subunits. These subunits are therefore expected to perform analogous functions in the enzyme complexes and may have highly conserved primary structures.

As an approach to the elucidation of the primary structure of oxaloacetate decarboxylase, the gene encoding this enzyme was cloned (175). Three *E. coli* clones capable of utilizing citrate as a sole carbon source were isolated from a cosmid bank of *K. pneumoniae* DNA. Two of these clones could grow on citrate only aerobically, but one could ferment citrate. In this clone the Na^+-activated, membrane-bound oxaloacetate decarboxylase was expressed. The gene for this enzyme has been subcloned on plasmids, and the determination of its primary structure is in progress. Since the synthesis of the decarboxylase is inducible by citrate

TABLE 3. Subunit composition of sodium transport decarboxylases

Decarboxylase	Organism	Molecular mass (kilodaltons)			
		α-Chain	β-Chain	γ-Chain	Biotin-carrier protein
Oxaloacetate	*Klebsiella pneumoniae*	65	34	12	α-Chain
	Salmonella typhimurium	63	34	10	α-Chain
Methylmalonyl-CoA	*Veillonella alcalescens*	60	33	14	18.5
Glutaconyl-CoA	*Acidaminococcus fermentans*	60	35	15 (?)	120, 140
	Peptostreptococcus asaccharolyticus	60	?		15
	Clostridium symbiosum	60	?		17, 20

(184), one can assume that a regulatory gene has been cloned as well (175). It is possible that the genes encoding citrate-lyase and citrate transport are linked to the structural genes for oxaloacetate decarboxylase and that an operonlike structure has been cloned (175).

Mechanism of decarboxylation reactions. During the decarboxylation of oxaloacetate (39), glutaconyl-CoA (21), or methylmalonyl-CoA (unpublished results), the stereochemical configuration is retained. In this respect these reactions are the same as all other investigated reactions catalyzed by biotin enzymes (218). All of the decarboxylases are completely inhibited by avidin and do not depend on a divalent metal ion, because ethylenediaminetetraacetic acid is without effect on them. The three decarboxylases are strongly activated by Na^+ ions, and oxaloacetate decarboxylase is completely inactive in the absence of Na^+ or Li^+ ions (50). The effect of Na^+ concentrations on initial velocity followed normal saturation kinetics (K_m, ≈1.5 mM) (50). Activation of oxaloacetate decarboxylase by Li^+ was less efficient (K_m, ≈25 mM; V, about 0.25 that in the presence of Na^+) (50). The K_ms of the other decarboxylases for Na^+ were also in the range of 1 mM (24, 80). Glutaconyl-CoA decarboxylase was also activated by Li^+ (K_m, ≈100 mM) (24). Potassium ions could not replace Na^+ or Li^+ in activating these enzymes.

Oxaloacetate decarboxylase was strongly inhibited by the substrate analogs glyoxylate, 2-oxomalonate, pyruvate, and especially oxalate (K_I, 3 to 4 μM), which is competitive with oxaloacetate (39, 50). The strong inhibitory effect of oxalate, with a K_I about 50 times lower than the K_m for oxaloacetate, indicates that this compound is a transition state analog. This correlates chemically with an almost identical spatial arrangement of atoms and distribution of charges in oxalate and the enolic form of pyruvate, the presumed transition state of the decarboxylation reaction (39). A structural comparison is shown in Fig. 6.

One question concerning the decarboxylation mechanism was whether H^+ or H_2O was the second substrate and whether CO_2 or HCO_3^- was the second product (equations 3 and 4).

$$R\text{-}COO^- + H^+ \rightarrow RH + CO_2 \quad (3)$$

$$R\text{-}COO^- + H_2O \rightarrow RH + HCO_3^- \quad (4)$$

The question was answered by performing the decarboxylation reactions at temperatures below 15°C, where the equilibration between CO_2 and HCO_3^- (equation 5) becomes rate limiting.

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons HCO_3^- + H^+ \quad (5)$$

When the pH of reaction mixtures containing oxaloacetate decarboxylase or methylmalonyl-CoA decarboxylase was followed with a glass electrode, a large alkaline overshoot was observed (48, 80). This overshoot was completely absent in the presence of carbonic anhydrase which accelerates the equilibration between CO_2 and HCO_3^- (equation 5). These results show that H^+ is the substrate and CO_2 is the primary product of the reaction.

The course of the reaction catalyzed by the biotin-dependent decarboxylases was presumed to involve the intermediate formation of an *N*-carboxybiotin enzyme derivative, like those formed in reactions catalyzed by all other biotin enzymes (Fig. 7) (146). In the first step, one would expect carboxylation of the biotin prosthetic group by carboxyl transfer from the substrate. Decarboxylation of the *N*-carboxybiotin enzyme intermediate would then regenerate the free biotin enzyme. Under physiological conditions the decarboxylation would be accompanied by the export of Na^+ ions from the cell.

The carboxyltransferase activity of oxaloacetate decarboxylase was determined by isotopic exchange between [1-^{14}C]pyruvate and oxaloacetate (44). The exchange reaction was sensitive to avidin as expected for a biotin-dependent process. Most importantly, Na^+ ions were not required for catalytic activity. The Na^+-dependent step in the catalytic cycle should therefore be the decarboxylation of the carboxybiotin enzyme intermediate. This supposition was shown to be true with $^{14}CO_2$-labeled carboxybiotin enzyme prepared by $^{14}CO_2$ transfer from [4-^{14}C]oxaloacetate in the absence of Na^+. When Na^+ was added, the enzyme-bound carboxyl group was rapidly released (44, 50). Similar isotopic exchange reactions have been observed with methylmalonyl-CoA decarboxylase (63) and glutaconyl-CoA decarboxylase (23). The activities of these carboxyltransferases were also independent of Na^+ ions (unpublished results) (23).

In view of the similarities in the subunit composition of the decarboxylases, one could suspect that the weight-homologous polypeptides are functionally related. To determine the function of the subunits, the complexes were dissociated and the subunits were isolated in a catalytically active state. The α-chain was dissociated from membrane-bound oxaloacetate decarboxylase by freezing and thawing in the presence of 1 M LiCl and purified to homogeneity by avidin-Sepharose affinity chromatography (48). The isolated α-chain was a catalytically active carboxyltransferase, but did not exhibit any oxaloacetate decarboxylase activity (48).

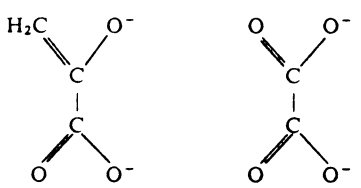


FIG. 6. Structural comparison of oxalate (right) and the enolic form of pyruvate (left).

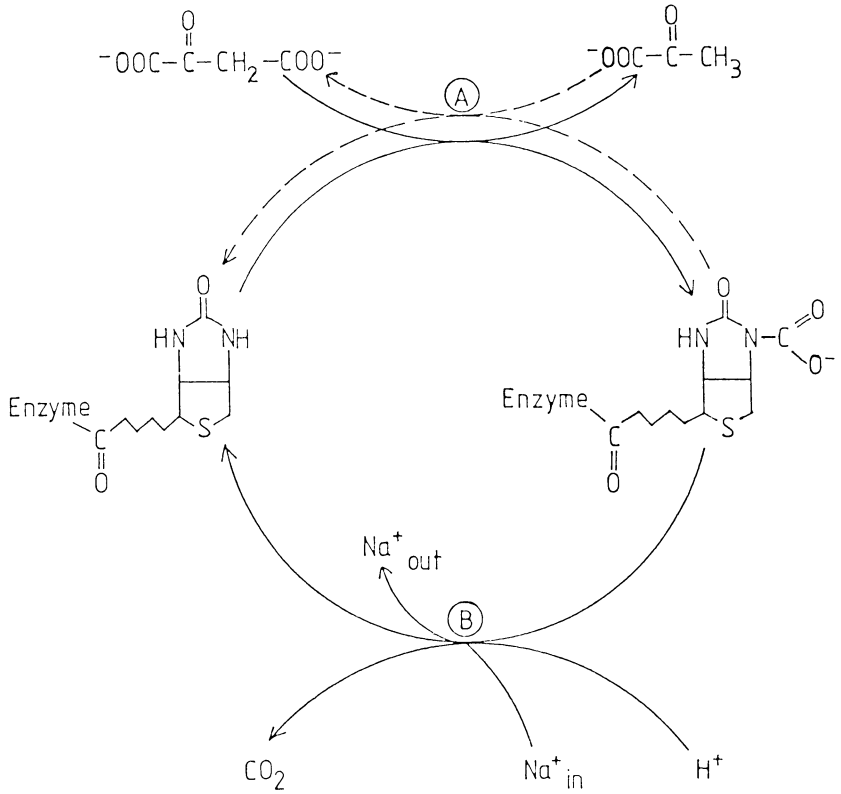


FIG. 7. Hypothetical mechanism of oxaloacetate decarboxylase. The carboxyltransferase activity is represented by reaction A, and the carboxybiotin enzyme decarboxylase activity is shown by reaction B. (Reproduced from reference 45 with permission.)

The carboxybiotin enzyme decarboxylase activity must therefore be contained on the β- or (β + γ)-subunit. The γ-chain alone is probably too small to act as a catalyst. The function of β + γ in the decarboxylation of the carboxybiotin enzyme was confirmed by restoring oxaloacetate decarboxylase activity from the isolated carboxyltransferase (α) and the isolated β- and γ-subunits (unpublished results).

When glutaconyl-CoA decarboxylase was incubated with small concentrations of a primary alcohol, e.g., 2% 1-butanol, the decarboxylase activity was inactivated, but some carboxyltransferase activity survived (23). Since the β-chain was specifically precipitated by the alcohol, the carboxyltransferase activity must reside in the larger soluble subunits of M_r 60,000 and 120,000 (Table 3). It is interesting that the inactivation of the β-chain was inhibited by NaCl (23). Previously it had been shown that Na^+ specifically protected the β-chains of all three decarboxylase complexes from tryptic hydrolysis (25, 48). Thus, this subunit has a binding site for Na^+ which, when occupied, forces the protein into a more resistant conformation. This notion is also in accord with the function of the β-chain in the Na^+-dependent step of the catalysis, i.e., the decarboxylation of the carboxybiotin enzyme. From the specific Na^+ requirement of this reaction step, one can further conclude that the β-chain is the protein that pumps Na^+ ions. This function also correlates with the strong hydrophobicity of the β-chain, a property characteristic of integral membrane proteins (23, 48). The carboxyltransferase subunits of oxaloacetate decarboxylase (48) and glutaconyl-CoA decarboxylase (23) are, on the other hand, soluble in the absence of any detergent, indicating that these are peripheral membrane proteins which extend mainly into the cytoplasm. The decarboxylases may thus have a gross structure similar to that of the F_1F_0 ATPase (74). The α-chain of oxaloacetate decarboxylase (Fig. 8) corresponds to F_1 and the β- and γ-subunits correspond to F_0. The arrangement of the subunits of the other decarboxylases in the membrane is probably analogous.

From a kinetic study of the reaction mechanism of oxaloacetate decarboxylase, it was concluded that the overall decarboxylation involves catalysis at two different and independent sites (50). Site 1 catalyzes the carboxyl transfer from oxaloacetate to enzyme-bound biotin. The carboxybiotin moves to site 2, where a proton is added and CO_2 is released, if this site is occupied with Na^+. The model derived from enzyme kinetics is thus in complete harmony with the results on subunit structure and function described above.

Reconstitution of Na^+ transport. To investigate the Na^+ transport activity of the decarboxylases, the purified enzymes were incorporated into liposomes by the detergent dilution method (162), using octylglucoside. Proteoliposomes carrying out Na^+ transport were prepared from soybean phospholipids and any one of the three decarboxylases under almost identical conditions (25, 40, 81). The shape of the proteoliposomes was investigated by electron microscopy (168). Proteoliposomes reconstituted with methylmalonyl-CoA decarboxylase were spherical, with a mean diameter of 62 nm, and each contained, on average, 9 to 10 enzyme molecules incorporated in the phospholipid bilayer. About 50% of the proteoliposomes became labeled upon incubation with avidin-gold complexes and contained, on average, 3.4 avidin-gold complexes on their surface. A comparison of the catalytic activity before and after disrupt-

ing the membrane barrier with Triton X-100 indicated that about 80% of the enzyme molecules were oriented with the substrate binding site facing the interior. With these data and the amount of protein incorporated, one could calculate that in each average-sized proteoliposome about one to two enzyme molecules are properly oriented to perform inwardly directed Na^+ pumping.

The kinetics of oxaloacetate decarboxylation and Na^+ uptake by reconstituted oxaloacetate decarboxylase-containing proteoliposomes are shown in Fig. 9 (45). Initially, close to 2 mol of Na^+ ions was pumped into the vesicles by decarboxylation of 1 mol of oxaloacetate. The stoichiometry changed when steeper Na^+ concentration gradients developed, because decarboxylation continued even after a constant internal Na^+ concentration was reached. Even then partial coupling persisted, because the decarboxylation rate increased about twofold after monensin was added, which completely abolished the Na^+ gradient. Similar results have been obtained with methylmalonyl-CoA decarboxylase reconstituted into liposomes (unpublished results). The energetic parameters of the Na^+ pump methylmalonyl-CoA decarboxylase were also determined with proteoliposomes. At its steady state the Na^+ gradient was 50 mV; the membrane potential was 60 mV and the total sodium motive force was thus equal to 110 mV. These values are very similar to those determined with oxaloacetate decarboxylase-containing bacterial vesicles (see above). To pump 1 mol of Na^+ ions against an electrochemical gradient of 110 mV requires 10.5 kJ. Since the free energy change of decarboxylation is ($\Delta G^{0\prime}$, ≈ -29 kJ/mol), it is sufficient to pump 2 to 3 mol of Na^+ per mol of substrate decarboxylation. The observed stoichiometry is therefore in accord with bioenergetic expectations.

Reversibility. Reversibility is recognized as a fundamental feature of coupled vectorial transport systems (93, 192). ATP-driven pumps (Ca^{2+}-, Na^+/K^+-, and H^+-ATPases) can perform either active transport of the appropriate cation by ATP hydrolysis or ATP synthesis at the expense of the cation gradient, the direction of operation depending on the free energy change under the conditions of the physiological steady state. The only known exception to the rule of reversibility is bacteriorhodopsin where the energy absorbed from light far exceeds the requirements of the transport process (187, 188). One would, therefore, expect that the decarboxylase systems are reversible also. Decarboxylation reactions are completely irreversible when catalyzed by the soluble enzymes (39, 63). To imitate the physiological situation in which the decarboxylations are coupled to Na^+ transport, the reversibility has been studied, using oxaloacetate decarboxylase and methylmalonyl-CoA decarboxylase reconstituted into proteoliposomes. According to equation 6, the reversibility could be measured by the isotopic exchange between $^{14}CO_2$ and the carboxylated substrate ($R\text{-}COO^-$).

$$R\text{-}COO^- + 2\ Na^+_{out} + H^+ \rightleftharpoons RH + CO_2 + 2\ Na^+_{in} \quad (6)$$

Proteoliposomes containing oxaloacetate decarboxylase catalyzed a $^{14}CO_2$-oxaloacetate exchange and methylmalonyl-CoA decarboxylase containing proteoliposomes catalyzed a $^{14}CO_2$-malonyl-CoA exchange (47). These exchange reactions must be completely dependent on the Na^+ ion gradients established during decarboxylation of part of the substrates because no exchange took place in the presence of monensin, which abolished the gradient. Net carboxylation of acetyl-CoA to malonyl-CoA was observed when a large inwardly directed Na^+ concentration gradient was applied to methylmalonyl-CoA decarboxylase-containing proteoliposomes; none was observed in the absence of a Na^+ gradient.

A transcarboxylase system was constructed by reconstituting oxaloacetate decarboxylase and methylmalonyl-CoA decarboxylase into the same liposomes (47). The Na^+ gradient developed by one of the enzymes functioning as decarboxylase could be used by the other enzyme to drive the carboxylation of its decarboxylated substrate (Fig. 10). This system catalyzed the carboxylation of acetyl-CoA to

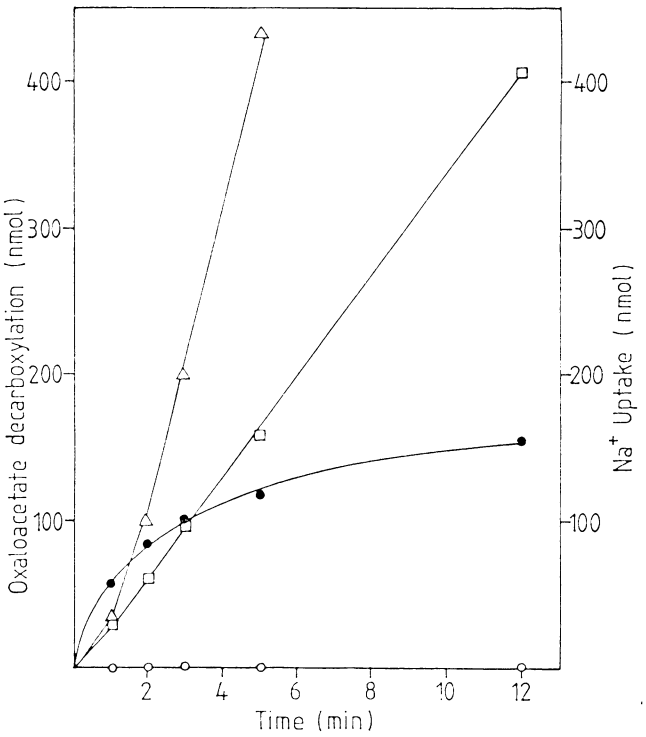


FIG. 9. Kinetics of oxaloacetate decarboxylation and Na^+ transport into reconstituted oxaloacetate decarboxylase-containing proteoliposomes. Symbols: Oxaloacetate decarboxylation (□); oxaloacetate decarboxylation in the presence of 0.2 mM monensin (△); Na^+ uptake (●); Na^+ uptake in the presence of 0.2 mM monensin (○). (Reproduced from reference 45 with permission.)

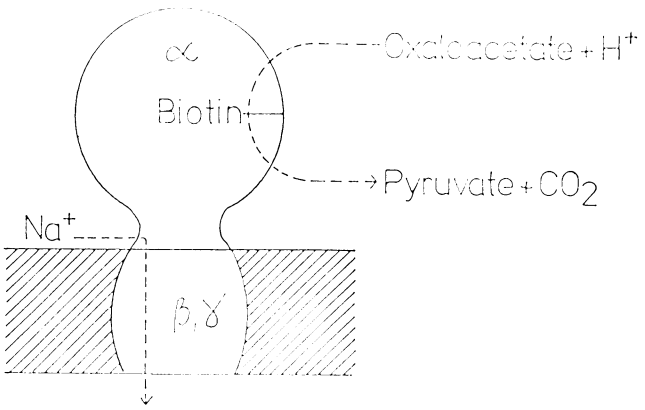


FIG. 8. Hypothetical model of the arrangement of oxaloacetate decarboxylase subunits in the membrane. (Reproduced from reference 48 with permission.)

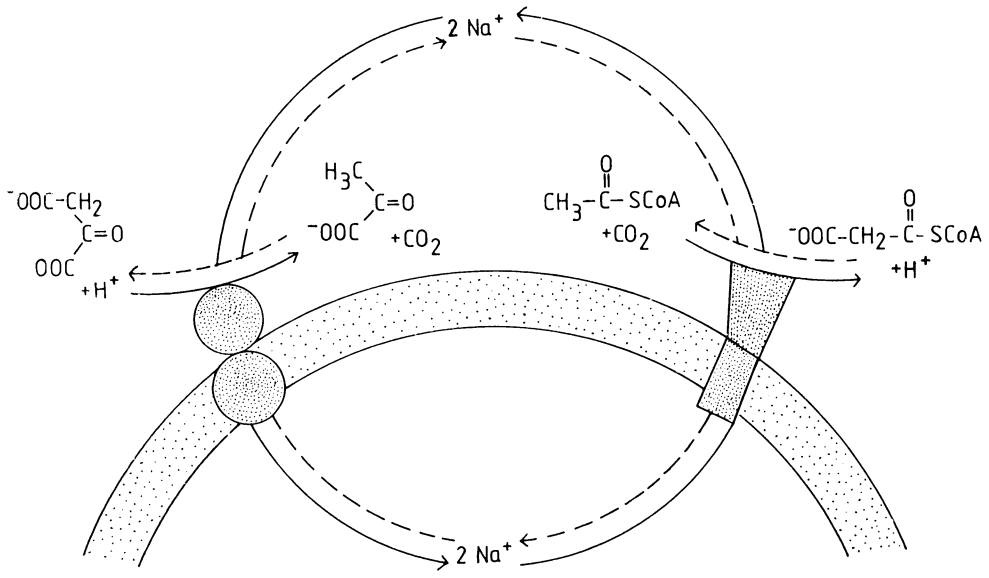


FIG. 10. Na^+ circuit mediating the transcarboxylation from oxaloacetate and acetyl-CoA to pyruvate and malonyl-CoA and vice versa. (Reproduced from reference 47 with permission.)

malonyl-CoA by decarboxylation of oxaloacetate to pyruvate and vice versa. The Na^+ circuit (Fig. 10) provides the energetic coupling between the exergonic decarboxylation and the endergonic carboxylation reaction. No carboxylation, therefore, occurred if the Na^+ gradient was dissipated with monensin. These are the first demonstrated examples of unfavorable carboxylation reactions being energized by an Na^+ gradient rather than by ATP hydrolysis.

The classical transcarboxylase of *Propionibacterium shermanii* (220, 221) catalyzes the same overall reaction as the membrane-linked transcarboxylase system constructed with the two decarboxylases. Moreover, carboxyltransferase and biotin carboxyl carrier protein components participate in both processes, but the coupling mechanisms are completely different. In soluble transcarboxylase, during the decarboxylation of the first substrate the carboxyl group is linked to the biotin prosthetic group from which it is directly transferred to the second substrate in the carboxylation reaction. Decarboxylation and carboxylation are thus coupled by an energy-rich chemical bond, the carboxyl group attached to biotin on the enzyme. This mechanism is distinct from the vectorial energy coupling in the membrane-linked transcarboxylase system. These two carboxylation mechanisms parallel the two mechanisms of ATP synthesis: substrate-level phosphorylation and phosphorylation coupled to vectorial H^+ movement.

Respiratory Na^+ Pump

Another primary bacterial sodium pump was detected by Tokuda and Unemoto (200, 201) during studies on the respiratory chain of *Vibrio alginolyticus*. An electrochemical Na^+ gradient was formed in this bacterium as a direct result of respiration. The respiratory Na^+ pump has a pH optimum at an alkaline external pH. At pH 8.5 the cells generate $\Delta\psi$ and ΔpNa^+, which are resistant to the uncoupler carbonylcyanide *m*-chlorophenylhydrazone (CCCP). The inside-negative $\Delta\psi$ drives CCCP-mediated H^+ ion movement into the cells, yielding a ΔpH of reversed sign (inside acidic) (200, 201).

Vibrio alginolyticus growing at alkaline pH was highly resistant to CCCP (202). This behavior was used by Tokuda (195) to select for CCCP-sensitive mutants that were found to lack the Na^+ pump activity. At neutral pH the mutants, like the wild type, generate $\Delta\psi$ and ΔpH by respiratory proton pumping and form ΔpNa^+ by the Na^+/H^+ antiporter. Membrane energization in these cells is, therefore, prevented by CCCP. The site of mutation was shown to be in a segment of the respiratory chain between NADH and quinol (203). The NADH oxidase in wild-type cells and spontaneous revertants required Na^+ and was inhibited by 2-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO), but the NADH oxidase in the mutants was independent of Na^+ and unaffected by HQNO (203). The function of NADH oxidase as an Na^+ pump was directly demonstrated by Na^+ accumulation in inverted bacterial vesicles and reconstituted proteoliposomes during NADH oxidation. Inhibition of NADH oxidase with HQNO prevented the accumulation of Na^+ (196, 199).

The Na^+-dependent site in NADH oxidation was shown to be the reduction of quinone to quinol, and this step was also shown to be the site for HQNO inhibition (203, 208). Hayashi and Unemoto (75) have isolated and partially purified the NADH:quinone oxidoreductase of *Vibrio alginolyticus*. The enzyme stoichiometrically reduced added quinone to quinol at the expense of NADH oxidation. This reaction specifically required Na^+ and was inhibited by HQNO (75). An NADH dehydrogenase was resolved from the NADH:quinone oxidoreductase and purified. This enzyme reduced the quinone by a single electron reaction, generating semiquinone radicals, which were readily autoxidized by molecular oxygen as revealed by the nonstoichiometric NADH oxidation and by the generation of superoxide radicals (75). Importantly, the NADH dehydrogenase was Na^+ and HQNO insensitive. The Na^+-dependent site in this system, therefore, appears to be the reduction of the semiquinone to quinol, which was suggested to be catalyzed by a dismutase that may function as the sodium pump (75) (Fig. 11).

Similar Na^+-motive NADH oxidases have been found in several other marine bacteria, e.g., *Vibrio costicola* (202, 207), *Vibrio parahaemolyticus* (205), and the halotolerant bacterium Ba_1 from the Dead Sea (101–103). The respiratory sodium pump may therefore be a general system for bacteria living in Na^+-rich environments. Avi-Dor and co-workers (101, 102) performed a detailed study on the respiratory sodium pump of Ba_1 with the result that the mechanism of coupling Na^+ transport to the respiratory chain appears to be very similar to that in *Vibrio alginolyticus*, although these two organisms are phylogenetically distinct. Sodium ions accelerated NADH oxidation by inverted membrane vesicles of Ba_1, the site of Na^+ action being the reduction of quinone to quinol. Results from electron-spin resonance spectroscopy indicated a signal tentatively identified as originating from semiquinone which increased on addition of NADH and decreased on addition of Na^+, as would be expected if Na^+ effected the semiquinone reduction (101) as proposed from different kinds of evidence for the *Vibrio alginolyticus* system (75).

A tentative mechanism of the respiratory Na^+ pump of marine bacteria (Fig. 11) involves two distinct steps: the Na^+-independent formation of the energy-rich semiquinone intermediate and its Na^+-dependent dismutation to quinol and quinone. The latter is the energy-releasing step and the one in which Na^+ translocation probably takes place. The overall construction of the respiratory Na^+ pump is, therefore, similar to the Na^+ transport decarboxylases and transport ATPases in that first a high-energy, enzyme-bound intermediate is formed in a reaction that is independent of the transported ion. Then in the following step the free energy liberated upon decomposition of the high-energy intermediate is used to drive the uphill transport of a specific ion.

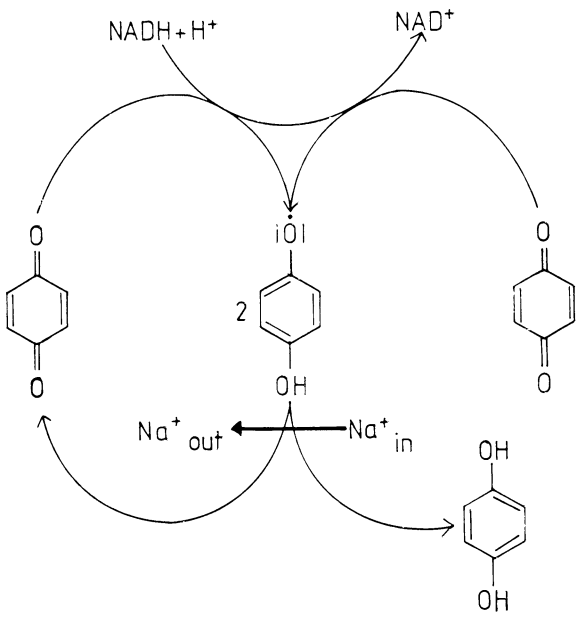


FIG. 11. Hypothetical mechanism of the respiratory Na^+ pump of marine bacteria.

Na^+ GRADIENT AS DRIVING FORCE FOR ENDERGONIC MEMBRANE REACTIONS

Na^+-Coupled Transport

Na^+-solute symport. Different citrate transport systems are expressed in *K. pneumoniae* when the bacteria are grown on citrate under aerobic or anaerobic conditions (49, 96, 215). The most prominent difference between these systems is a specific requirement for Na^+ or Li^+ by the anaerobic citrate transport system. Neither of these ions is essential for the aerobic system (49). The potential of *K. pneumoniae* to express different citrate uptake systems is in accord with molecular genetic studies from which the existence of at least three different citrate transport proteins has been postulated (175). There are apparent strain variations within the *K. pneumoniae* group because citrate uptake by some strains was reported to be potassium dependent (53). Multiple citrate transport systems with different requirements for Na^+ and K^+ also exist in *Salmonella typhimurium* (4, 100). In at least one of these, a periplasmic binding protein is involved; this protein has been isolated (190). The binding of citrate to this protein was enhanced by Na^+ and slightly inhibited by Mg^{2+}, Zn^{2+}, or Co^{2+} (191). Another variation of citrate uptake was found in *Bacillus subtilis*; here the tricarboxylic acid is cotransported with a divalent metal ion, e.g., Mg^{2+} (11, 216), and probably protons (11). Thus, there exists a great diversity of citrate uptake systems in bacteria, Na^+-dependent citrate transport being a rather rare case, probably restricted to organisms containing a decarboxylase Na^+ pump.

The energetic requirements of the anaerobic citrate transport system of *K. pneumoniae* have been studied with whole cells and vesicles (49, 96). In addition to the dependence on Na^+, citrate accumulation was also dependent on both components of the proton motive force, $\Delta\psi$ and ΔpH (49). Citrate uptake, therefore, appears to be an electrogenic process, which implies that the tricarboxylate must enter the cell in symport with at least four positive charges. Based on several lines of evidence (49), these cotransported ions are probably Na^+ and H^+. These cations must be exported again to complete the cycle, which poses a severe bioenergetic problem for growth under anaerobic conditions in which only 1 mol of ATP is obtained per mol of citrate catabolized (cf. Fig. 2). This yield of ATP would not even be sufficient to recycle the protons and metal ions taken up with citrate assuming the following stoichiometries: $(H^+ + Me^+)$:citrate = 4; $(H^+ + Me^+)$:ATP = 3. A model for citrate transport and the recycling of the symported cations by an ATP-independent mechanism that we have recently proposed (49) is shown in Fig. 12. Since oxaloacetate decarboxylase pumps two Na^+ ions out of the cell, two Na^+ ions could be taken up together with citrate. The two protons that then must be cotransported could leave the cell with an extrusion of the metabolic end products acetate, formate, and bicarbonate in their protonated form. This mechanism accounts for the proton recycling and generation of ΔpH, at the expense of the chemical gradients of end products; ΔpH could drive citrate accumulation (49).

Konings and co-workers (138) were the first to propose that fermenting organisms may conserve energy by taking advantage of their large amounts of metabolic end products. If an end product such as lactate is exported by a carrier-mediated symport with two protons, an electrochemical proton gradient would form at the expense of the lactate gradient, the mechanism being an inverse of H^+-coupled solute uptake. This hypothesis was experimentally verified when deenergized cells of *S. cremoris* were shown to accumulate leucine in response to an efflux of lactate (158). The

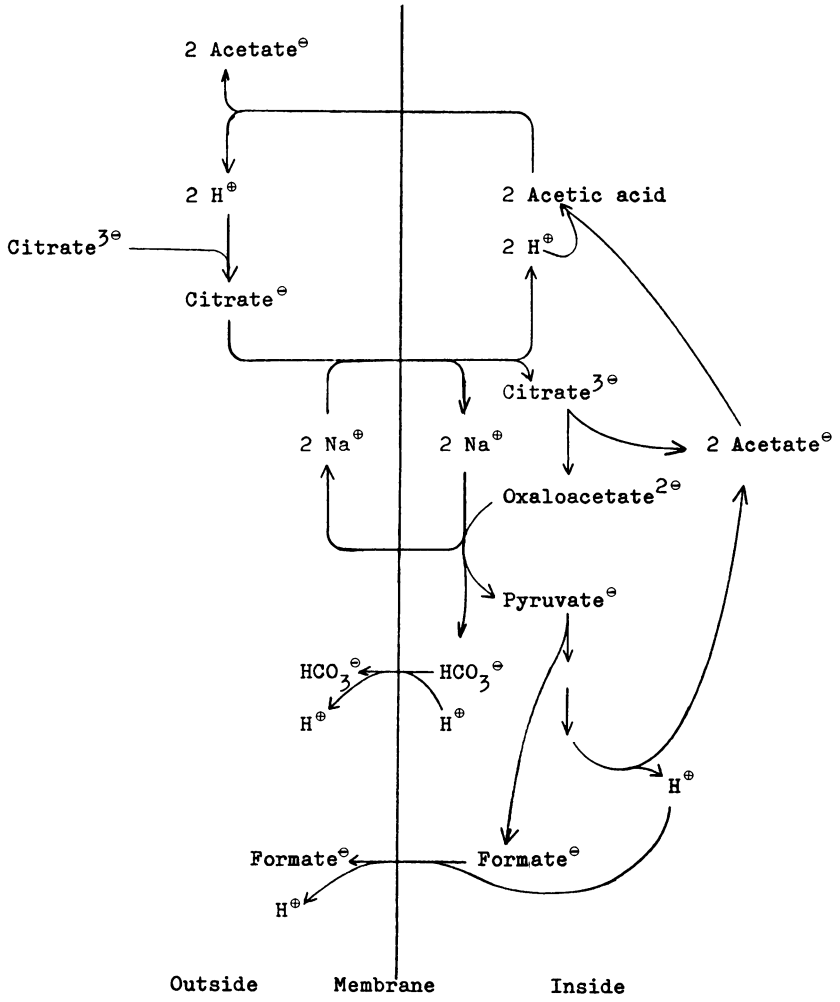


FIG. 12. Hypothetical model of ion fluxes in citrate-fermenting *K. pneumoniae*. (Reproduced from reference 49 with permission.)

utilization of this energy source was also demonstrated by a considerable increase of the molar growth yield of *S. cremoris* growing on lactose at low external lactate concentrations (157).

The above considerations of ion movements in *K. pneumoniae* may suggest why decarboxylation-dependent energy-converting systems use Na^+ and not H^+ as the coupling ion (49). If in *K. pneumoniae* an H^+ gradient were formed by oxaloacetate decarboxylation, it would counteract the extrusion of fermentation products symported with protons with the consequence of forming high, possibly intolerable, internal concentrations of these acids. It is possible that under such conditions extrusion of fermentation products would proceed uncoupled from proton extrusion; it would then not contribute to ΔpH generation. A decarboxylase pumping H^+ instead of Na^+ would thus lead to a diminution of the total energy conserved.

A few other transport systems that are coupled to Na^+ ions have been characterized in enterobacteria. Glutamate transport in *E. coli* was shown to be stimulated by Na^+; Na^+ increased the affinity for the substrate (58). Based on the observation that glutamate was accumulated with an imposed Na^+ gradient, a glutamate/Na^+ symport mechanism was proposed (73, 130). Since ΔpNa^+ as well as ΔpH provided a driving force for the transport, the model was extended by postulating that glutamate transport occurred by syn-coupling with Na^+ and H^+ (59). This would be very similar to our proposed mechanism of citrate transport by *K. pneumoniae* (49). Further insight into the mechanism of glutamate transport was obtained by binding studies showing that glutamate binding to the carrier occurs only if it is loaded with Na^+ and H^+ ions (60). Proline transport has been generally considered to be energized by a proton symport mechanism, but recently it was recognized that the major proline uptake systems of *E. coli* and *Salmonella typhimurium* are coupled to a cotransport with Na^+ ions (26, 29, 185). Among the various mechanisms for sugar transport in bacteria, an Na^+-coupled transport exists in the melibiose uptake system of *E. coli* and *Salmonella typhimurium* (186, 197, 204, 206). The melibiose carrier of *E. coli* has unique cation coupling properties. This system is capable of using H^+, Na^+, and Li^+ as coupling cations depending on the particular sugar being transported (149, 206). It was shown by binding studies that Na^+ (or Li^+) ions selectively increased the apparent affinity of the porter for galactosides whereas protons apparently competitively inhibited this Na^+ activation (32). A two-branch model was proposed in which the carrier is first loaded with either Na^+ or H^+, after which the ternary complex with the substrate is formed and translocation takes place (32). It is interesting that the ability of H^+-coupled melibiose uptake could be impaired by a simple point mutation that causes the transport to be re-

stricted to symport with Na^+ or Li^+ (149). More recently, the *melB* gene which codes for the melibiose carrier in *E. coli* has been cloned (71) and sequenced (222). Insight on the molecular level into the interesting phenomenon of coupling to various cations can now be anticipated. An alanine carrier that is composed of a single polypeptide, M_r 42,500, was isolated from the thermophilic bacterium PS3 (84). This protein is another example of carrying out an active transport driven by an electrochemical potential difference of either protons or sodium ions. *Pseudomonas aeruginosa* (88) and *B. subtilis* (118), bacteria that occur in Na^+-poor environment, also contain sodium-dependent transport systems for single amino acids.

Bacteria from Na^+-rich habitats use symport with Na^+ ions as the predominant or exclusive mechanism for amino acid uptake (119). Lanyi et al. (121) and MacDonald et al. (129) have studied amino acid transport by *H. halobium*. Using envelope vesicles, they demonstrated that the driving force for transport is an electrochemical gradient of Na^+ ions. An Na^+-dependent transport of a variety of amino acids is also a well-established feature in the marine bacteria *Alteromonas haloplanctis* (56, 131, 151, 182, 183, 194), *Vibrio costicola* (70), and *Vibrio alginolyticus* (198, 201). In the latter organism evidence for Na^+-sucrose symport has also been obtained (98). This predominance of Na^+ cotransport systems is shared by the obligate alkalophiles. Thus, transports of malate, α-aminoisobutyrate, glutamate, and several other amino acids were all found to be Na^+ dependent (13, 68, 113). Mutants of *B. alcalophilus* and *B. firmus* have been isolated that were unable to grow at alkaline pH, because they suffered a defect in the Na^+/H^+ antiporter, and thereby the pH homeostasis mechanism was impaired (67, 68, 116, 124). Also, the Na^+ coupling for several solutes was pleiotropically lost, and H^+ served instead as the coupling ion for these symport systems. A similar mutant of *E. coli* was described by Zilberstein et al. (225) with defects in three different Na^+-coupled transport systems: Na^+/H^+ antiport, Na^+/glutamate symport, and Na^+/melibiose symport. The existence of mutants with a pleiotropic defect of several Na^+-coupled porters suggests that a common gene is responsible for the function of these systems. This gene could either code for a common Na^+-translocating subunit (68) or, more likely (115), have a regulatory function.

In summary, the Na^+-coupled symporters of bacteria can be divided into three groups. In the first group, represented by glutamate uptake in *E. coli* and citrate transport in *K. pneumoniae*, both Na^+ and H^+ are symported with the solute. In the second, represented by the melibiose carrier of *E. coli* and various permeases of alkalophiles, the transport can be coupled to either Na^+ or H^+ ions, and by a simple mutation the recognition of the carrier for one of these cations can be impaired. Examples of the third group are the major proline transport systems of *E. coli* and *Salmonella typhimurium* which exclusively use Na^+ as coupling ion. Wilson and Maloney (217) have speculated that during evolution the proton currency for membrane energy transductions, being the most primitive form, has changed to the Na^+ energetics of the plasma membranes of animal cells. It was proposed that the melibiose carrier might be a descendent of a transport protein that appeared during the period of transition between H^+ and Na^+ membrane economies (217). This idea can be extended to other groups of the Na^+-coupled symporters in bacteria which may represent different stages in the evolution from H^+ to Na^+ bioenergetics.

Na^+/H^+ antiport. Another important Na^+ transport system of bacteria is the Na^+/H^+ antiporter (111, 119, 211). Since bacteria do not generally contain a primary Na^+ pump, one physiological function of the antiporter is to generate the Na^+ ion gradients for the Na^+-symport processes. The antiporter uses the proton motive force generated by a proton pump and converts this into an electrochemical Na^+ gradient. A second function of the Na^+/H^+ exchanger is to regulate cytosolic pH (14, 159), especially in the obligate alkalophiles that live in environments of pH 9 to 11 and maintain a cytoplasmic pH of <9.5 (113). In accord with this notion, growth of *B. firmus* on glucose required Na^+ at a medium pH of >9.7 (114). Krulwich and colleagues (106) have observed that, upon dilution of a culture of *B. firmus*, growing at pH 10.5, into an Na^+-free medium, the cytoplasmic pH immediately rose to pH 10.5 and viability was rapidly lost. The ΔpH generated by respiring cells or membrane vesicles of *B. firmus* at pH 9.0 was acid outside in the absence of Na^+ and acid inside in the presence of Na^+ (106, 114). This Na^+ dependence of the development of a pH gradient, acid inside, was also observed in *Exiguobacterium aurantiacum*, another alkalophilic bacterium (137). Thus, acidification of the cytoplasm at alkaline environmental pH is strictly Na^+ dependent, suggesting that the Na^+/H^+ antiporter is responsible for pH homeostasis. Accordingly, the defect in nonalkalophilic mutant strains which were unable to grow at a pH of >9.0, could be attributed to the Na^+/H^+ exchange activity (67, 116). The driving force for antiport activity was contributed by the membrane potential, which might indicate that the Na^+-H^+ exchange is electrogenic with $H^+ > Na^+$ (65). Important for the function of the Na^+/H^+ exchanger in pH homeostasis is a striking effect of internal pH on antiport activity which increased about 10-fold from pH 7 to 10 (65). The importance of Na^+ for pH homeostasis in alkalophiles was further demonstrated by the improved acidification of the cytoplasm in the presence of a solute that is symported with Na^+ (112). A sodium entry route is thus provided and the Na^+ cycle is closed. In some species of the alkalophiles, in which a dependence on Na^+ for growth and acidification of the cytoplasm could not be directly demonstrated, the affinity of the antiporter for Na^+ was unusually high; thus, the unavoidable contaminating Na^+ concentration might be sufficient for these functions (114).

The exact role of the Na^+/H^+ exchanger for pH homeostasis in neutrophilic bacteria is a matter of debate (14, 159, 224). Such a function could be predicted from recent findings of Bassilana et al. (6) that the activity of the antiporter is regulated by the cytosolic pH, but from other work it was suggested that a K^+/H^+ antiporter is involved in regulating cytosolic pH in *E. coli* (18) or *Vibrio alginolyticus* (147). The other important function of the Na^+/H^+ antiporter, i.e., generation of an Na^+ gradient for symport processes, however, is not disputed for most bacterial cells because, with the exception of some species which contain a primary Na^+ pump, bacteria need the antiporter for $\Delta\tilde{\mu}Na^+$ generation. Especially for halobacteria, which perform most of their solute uptake as cotransport with Na^+, the generation of an Na^+ gradient is a critical event (119). An Na^+/H^+ antiporter is well documented in *H. halobium* which uses $\Delta\tilde{\mu}H^+$ established by bacteriorhodopsin as the driving force for Na^+ extrusion (120–122). In salty environments (e.g., 4 M NaCl) large movements of Na^+ are necessary to create a significant concentration gradient (the internal Na^+ concentration is maintained between 0.3 and 2 M), and for the same reasons the Na^+ gradient provides a large energy reservoir

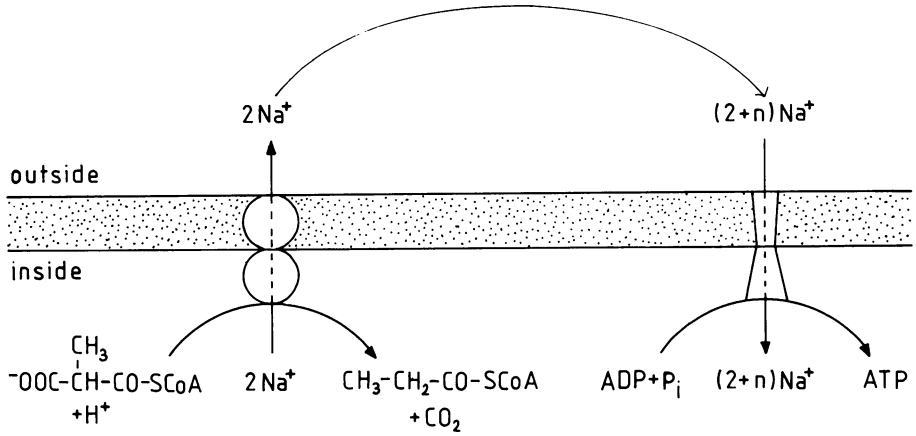


FIG. 13. Na^+ cycle mediating ATP synthesis by methylmalonyl-CoA decarboxylation in *P. modestum*. (Reproduced from reference 83 with permission.)

for the cells (19). The mechanism of the antiporter of *H. halobium* was found to be an electrogenic exchange with an H^+:Na^+ stoichiometry of 2:1, which means that the driving force for Na^+ export can be provided by ΔpH and $\Delta\psi$ (122). The electrogenic mechanism is especially advantageous at alkaline pH when ΔpH is zero or negative and an uphill extrusion of Na^+ is only possible by coupling to $\Delta\psi$. Another example of an electrogenic Na^+/H^+ antiporter exists in *E. coli* (6–8, 174). The electrogenic mechanism may thus be of general importance for bacteria. The Na^+/H^+ antiporter of *H. halobium* was also found to be gated, requiring a $\Delta\tilde{\mu}H^+$ of -130 to -150 mV for significant activity (122). This gating may be physiologically important to prevent a massive influx of Na^+ in starved cells with a low $\Delta\tilde{\mu}H^+$ by reversal of the antiporter function (122).

Na^+-Driven Flagellar Motors

It is now well established that the flagellar motor of bacteria living under moderate environmental conditions is powered by proton motive force (66, 132). For optimal rotation of these motors rather large $\Delta\tilde{\mu}H^+$ values (-80 to -100 mV) are required (104, 135). Since alkalophilic bacteria have to acidify the cytoplasm, their proton motive force is low, especially at pH 10 to 11 (67), where at least some alkalophilic *Bacillus* strains are highly motile (86). The bioenergetic problem was solved by Imae's group (86, 87, 189) when these investigators showed that motility was coupled to the electrochemical Na^+ gradient like many solute transport systems in these bacteria. The motility of alkalophilic *Bacillus* sp. strain YN-1 at pH 9 was strictly dependent on Na^+, and the swimming speed increased at increasing Na^+ concentrations until a plateau was reached at about 100 mM NaCl (86, 87). The energy for flagellar motion was provided by both membrane potential and Na^+ concentration gradient, and a threshold value for motility was found at a $\Delta\tilde{\mu}Na^+$ of about -100 mV (86).

The motility of *B. firmus* (106) and *Vibrio alginolyticus* (30, 36) was also dependent on the presence of sodium ions. Swimming of *Vibrio alginolyticus* required an Na^+ concentration gradient and is most probably powered by the respiratory Na^+ pump contained in this organism (30, 36).

Na^+-Coupled ATP Synthesis and Alkali Ion-Translocating ATPases

The first indication of an Na^+-coupled ATP synthesis was obtained in the strictly anaerobic bacterium *P. modestum* (83). As described above, decarboxylation of methylmalonyl-CoA is the only energy-yielding reaction when this bacterium grows on succinate. Since this decarboxylation is associated with Na^+ pumping, the resulting Na^+ gradient provides the only energy source for ATP synthesis. In principle, ATP could be synthesized in *P. modestum* by two different mechanisms. Either the Na^+ gradient is first converted into a proton gradient through an Na^+/H^+ antiporter and ATP synthesis is driven by a H^+ gradient or a novel Na^+-dependent ATP synthase uses the Na^+ gradient directly to drive ATP synthesis. It has been shown by Hilpert et al. (83) that the membranes of *P. modestum* contained large amounts of an ATPase that was specifically activated by Na^+ ions. Furthermore, inverted membrane vesicles accumulated Na^+ in response to ATP hydrolysis. The sodium ion transport was abolished by monensin but was not significantly affected by trifluoromethoxycarbonylcyanide *p*-phenylhydrazone, indicating a direct coupling of Na^+ transport with ATP hydrolysis and none via the intermediate formation of a proton gradient. It was also shown that the two sodium pumps of *P. modestum* are energetically coupled (Fig. 13). The membrane vesicles thus catalyzed both monensin-sensitive ATP synthesis by malonyl-CoA decarboxylation and acetyl-CoA carboxylation by ATP hydrolysis. The rates of these transport-coupled syntheses and the rates of Na^+ transport were low in comparison to the rates of ATP hydrolysis or malonyl-CoA decarboxylation. The bacterial membrane system is thus highly uncoupled and not well suited to study these Na^+ ion gradient-coupled processes. More recently, the ATP synthase has been isolated and incorporated into liposomes with reconstitution of ATP-dependent Na^+ accumulation. This was not inhibited, but rather stimulated by CCCP; thus, functioning of the ATP synthase as a primary Na^+ pump is firmly established. A relationship of the *P. modestum* ATP synthase with F_1F_0 ATP synthases has also been demonstrated; the enzyme was strongly inhibited by *N,N'*-dicyclohexylcarbodiimide but not by vanadate. It consisted of a soluble, catalytic part with the typical subunit pattern of F_1 ATPases and a membrane-bound F_0 part that apparently contains the Na^+-binding site, because activation of ATPase activity by Na^+ was dependent on F_0 (unpublished results). To my knowledge *P. modestum* is the first organism for which ATP synthesis was shown to be coupled to an Na^+ gradient rather than to H^+ circulation.

Very recently, Skulachev and colleagues (37) have re-

ported Na^+-dependent ATP synthesis by whole cells of *Vibrio alginolyticus*. By applying a pulse of Na^+ to these cells, a temporary increase in the cellular ATP level was observed which was resistant to CCCP and abolished by monensin. Furthermore, ATP synthesis driven by the oxidation of lactate was prevented by applying ΔpNa^+ of reversed sign. Oxidative phosphorylation was resistant to CCCP and arrested by CCCP plus monensin, whereas monensin alone had no effect. *Vibrio alginolyticus* may thus be another organism in which ATP synthesis is accomplished by the Na^+-coupled mechanism.

This type of Na^+-coupled ATP synthesis may affect our thinking about ATP synthesis mechanisms in general. Mitchell (143, 145) proposed a mechanism in which the vectorial movement of protons is integrated with the chemical events at the catalytic site. In this model, the protons flow down the electrochemical gradient through F_0F_1 into the catalytic site, where they attack one of the oxygen atoms of phosphate, forming H_2O and $H_2PO_3^+$ which can directly react with ADP to form ATP. Since this mechanism is obviously restricted to protons as coupling ions, it is disfavored by the detection of an Na^+-coupled ATP synthase. However, the alternative suggestion of Boyer (16) that the energy available from the electrochemical gradient is used to promote substrate binding and product release by a distinct set of conformational changes is in accord with either H^+- or Na^+-coupled ATP synthesis.

A bioenergetic problem confronting *P. modestum* is the low amount of energy liberated by decarboxylation of methylmalonyl-CoA ($\Delta G^{0\prime} \approx -27$ KJ/mol), an amount that is not sufficient to support the synthesis of 1 mol of ATP (83). One must assume, therefore, that more than 1 mol of methylmalonyl-CoA is decarboxylated to synthesize 1 mol of ATP and, thus, that the two membrane-linked processes are coupled to different stoichiometries of Na^+ ions. Since two Na^+ ions are pumped out by the decarboxylase, at least three must enter the cell during ATP synthesis. It is clear from these considerations that the smallest quantum of biologically useful energy is not the free energy of ATP hydrolysis, but that of an ion forming a gradient across a membrane. The mechanism of ATP synthesis in *P. modestum* has significance for our understanding of the life of a number of other microbes that grow from fermentations with a free energy span insufficient to synthesize 1 mol of ATP per fermentation cycle. Examples are the anaerobic decarboxylation of oxalate to formate (33) and the formation of methane from acetate (133, 223).

There are a number of other reports on alkali ion-translocating ATPases in bacteria. Jinks et al. (94) described a fourfold stimulation of ATPase activity by Na^+ in *Acholeplasma laidlawii* and concluded that the enzyme translocated Na^+ across the membrane. Although the enzyme was subsequently purified by the same group, the validity of this concept was not further substantiated (125). The bioenergetics of alkali ion movements have also been studied in two other mycoplasmas. Benyoucef et al. (9, 10) investigated Na^+ and K^+ transport in *Mycoplasma mycoides* var. Capri cells. The uptake of K^+ by these cells was stimulated by Na^+, and the extrusion of Na^+ was stimulated by K^+. It was proposed that the organism may contain an Na^+/K^+ ATPase like the plasma membrane ATPase of higher organisms (10). The activity of the ATPase of *M. mycoides* was stimulated by Na^+ but not by K^+, although Na^+ export against a concentration gradient specifically required the presence of K^+ (10). In contrast, the activity of the ATPase of *M. gallisepticum* was only slightly stimulated by Na^+ and K^+ ions (37% at maximum) and the effect was not specific, since each monovalent cation slightly increased the catalytic rate (126). The experimental evidence points to Na^+ movements in this organism by the combined action of a proton-translocating ATPase and an Na^+/H^+ antiporter (127).

There is also evidence for the existence of an Na^+-translocating ATPase in *S. faecalis*. Heefner and Harold (76, 77) found a small but specific stimulation of ATP hydrolysis by Na^+ ions which correlated with ATP-dependent Na^+ transport into bacterial membrane vesicles. The activities of the Na^+-stimulated ATPase and of Na^+ transport were extremely low. However, in a mutant of *S. faecalis*, defective in proton-translocating ATPase, Kinoshita et al. (105) observed about 40-fold higher activity of the Na^+-stimulated ATPase. More recently, Kakinuma and Harold (97) have investigated the properties of KtrII, a potassium transport system of *S. faecalis* that was detected by Kobayashi (107). They found that K^+ accumulation by the bacterial cells required intracellular Na^+ ions and that K^+ was required for Na^+ extrusion, which may indicate an Na^+/K^+ exchange system (97). From other evidence it was proposed that the Na^+-stimulated ATPase in this organism is identical to KtrII and catalyzes an exchange of internal Na^+ for external K^+ by ATP hydrolysis (97). Thus, *S. faecalis* and *M. mycoides* may have similar types of Na^+/K^+ ATPase.

It should be noted that an additional K^+ transport system, named KtrI, exists in *S. faecalis* (5, 97) and that a potassium-translocating ATPase has been purified from this organism (89) that probably is identical to KtrI (61). There are also two different K^+ transport systems in *E. coli*. The KtrI system of *S. faecalis* parallels the TrK system of *E. coli* (166), but whether or not KtrII and Kdp (54, 55) are also analogous remains to be shown (97). In summary, evidence from quite distinct organisms indicates a great diversity of ion-translocating ATPases in bacteria. In addition to H^+ translocation, bacterial ATPases can perform Na^+ or K^+ movements and possibly the exchange of Na^+ for K^+.

Some Related Observations

Several other bacteria require Na^+ for special functions, but the exact role of Na^+ in these cells is only beginning to emerge. In the cyanobacterium *Synechococcus leopoliensis* Na^+ not only was required for pH homeostasis at alkaline external pH, but also had some function in cell division (139). The cells were able to photosynthesize normally at pH 6.8 in an Na^+-free medium, but were unable to divide and thus enlarged to about twice their normal size (139). In *Anabaena variabilis*, another cyanobacterium, Na^+ addition markedly increased the rate of photosynthesis, and it was proposed that the Na^+ affected the transport of bicarbonate into the cells (99).

Growth and methane formation from CO_2 in *Methanobacterium thermoautotrophicum* was dependent on the presence of low concentrations of Na^+ (160), and Na^+ also stimulated methane formation in several other species of methanobacteria (161). The Na^+-dependent step in methane formation from methanol by *Methanosarcina barkeri* was shown to be the oxidation of methanol to the oxidation level of formaldehyde (12). In this organism, Na^+ was not required for methane formation from methanol plus H_2 or methanol plus formaldehyde; neither was it required for the synthesis of ATP (12). In contrast, ATP synthesis in *Methanobacterium thermoautotrophicum* induced by an artificially generated membrane potential was greatly stimu-

lated by Na^+ (173). The mechanism of Na^+ action in ATP synthesis, however, is not clear, because no Na^+-stimulated ATPase was detectable (172). The presence of an Na^+/H^+ antiporter in *Methanobacterium thermoautotrophicum* has been reported (172). In *Methanococcus voltae*, ATP synthesis driven by a potassium diffusion potential was also Na^+ dependent and an Na^+ gradient alone was sufficient to drive ATP synthesis in the presence of tetraphenylborate (28). However, since the amount of ATP synthesized was low and since the results were obtained with whole cells, the existence of an Na^+-translocating ATPase in *Methanococcus voltae* is dubious. The exact role of Na^+ in ATP synthesis by methanogenic bacteria therefore remains to be determined.

Sodium and potassium ion gradients also serve as important energy reservoirs of bacterial cells (51, 178, 210). The first indication for this type of energy storage mechanism came from studies on *H. halobium* cells in which the capacity of the alkali ion gradients is at its maximum. During illumination of *H. halobium* K^+ ions were accumulated in the cytoplasm in response to the membrane potential created by bacteriorhodopsin, and Na^+ ions were extruded by the Na^+/H^+ antiporter (210). During the dark period these reactions were reversed, and by the continuous regeneration of $\Delta\psi$ due to electrogenic K^+ efflux the ATP level was stabilized (210). By measuring motility, $\Delta\psi$, or ATP levels, as an expression of the proton motive force, it was subsequently found that different bacteria were able to use Na^+ and K^+ gradients for $\Delta\mu H^+$ generation after other energy sources were exhausted (19). These interconversions of an electrochemical H^+ gradient and alkali ion gradients provide a means of greatly increasing the energy storage capacity of a bacterial cell (19, 178, 210). The amount of energy that can be stored in the form of $\Delta\tilde{\mu}H^+$ alone is low due to limitations in the electrical capacity of the membrane and due to the narrow tolerance of cytoplasmic pH values for viability. The monovalent metal ion gradients thus serve the function of a $\Delta\tilde{\mu}H^+$ buffer, the capacity of which is proportional to the content of the alkali ions in the environment. The greatest amount of energy could thus be stored in *H. halobium*; the smallest capacity was observed in the freshwater cyanobacterium *Phormidium uncinatum* from Lake Baikal (19).

CONCLUDING REMARKS

A large body of evidence has accumulated during the last decade that proton circuits, although of major importance, are not unique in the bioenergetics of bacteria. It is now clear that the coupling mechanisms are more diverse than originally anticipated, specifically accommodated to the physiological situation of the bacteria with respect to metabolism and habitat. Sodium ions have been recognized as the principal additional coupling ions that mediate exergonic and endergonic reactions in the membrane. Some bacteria perform all of their membrane bioenergetic tasks with proton cycles, others have sodium cycles in addition, and still others may perform all of their work by Na^+ cycling. Several advantages of using Na^+ cycling in addition to or instead of H^+ cycling can be anticipated. (i) Na^+ is a better coupling ion at alkaline pH at which the proton gradient is reversed to protect cellular components from denaturation. (ii) Na^+ gradients have a much higher energy storage capacity than proton gradients, at least in marine and halophilic bacteria. (iii) By adding an Na^+ cycle to a proton cycle, an important control element can be introduced. An example may be *H. halobium* in which the Na^+-coupled symporters only work at a significant rate above a certain level of $\Delta\tilde{\mu}H^+$ due to the gating of the Na^+/H^+ antiporter, whereas H^+-coupled ATP synthesis can still be operating reasonably well. (iv) Fermenting organisms with H^+-coupled extrusion systems for metabolic end products will create ΔpH by this mechanism. In the presence of an existing H^+ gradient generated by a proton pump, end-product extrusion may proceed without energy conservation; an Na^+ pump, however, would not interfere. Thus, an improved energy economy under certain conditions and the advantages of a more sophisticated control of different bioenergetic reactions may have led to the development of sodium bioenergetics in addition to proton bioenergetics during evolution.

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